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A Nuclear Hormone Receptor Corepressor Mediates Transcriptional Silencing by Receptors with Distinct Repression Domains

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Ligand-independent transcriptional repression is an important function of nuclear hormone receptors. An interaction screen with the repression domain of the orphan receptor RevErb identified N-CoR, the corepressor for thyroid hormone receptor (TR) and retinoic acid receptor (RAR). N-CoR is likely to be a bona fide transcriptional corepressor for RevErb because (i) RevErb interacts with endogenous N-CoR, (ii) ectopic N-CoR potentiates RevErb-mediated repression, and (iii) transcriptional repression by RevErb correlates with its ability to bind N-CoR. Remarkably, a region homologous to the CoR box which is necessary for TR and RAR to interact with N-CoR is not required for RevErb. Rather, two short regions of RevErb separated by ~200 amino acids are required for interaction with N-CoR. The primary amino acid sequence of the N-terminal region of RevErb essential for N-CoR interaction is not homologous to that of TR or RAR, whereas similarities exist among the C-terminal domains of the receptors. N-CoR contains two adjacent but distinct interaction domains, one of which binds tightly to both RevErb and TR whereas the other binds more weakly and differentially interacts with the nuclear receptors. These results indicate that multiple nuclear receptors, utilizing different primary amino acid sequences, repress transcription by interacting with N-CoR.

Regulation of gene expression is essential for cellular differentiation, development, and maintenance of homeostasis. These processes are regulated at the transcriptional level by sequence-specific transcriptional activators and repressors which communicate with the basal transcription apparatus (for reviews, see references 51 and 59). Many activation domains have been shown to interact directly with components of the basal transcription apparatus, while others interact indirectly by contacting intermediate proteins termed adapters or coactivators. Activation domains have been classified by the amino acid residues that predominate: acidic (29, 38), glutamine rich (12), or proline-rich (41). It has been hypothesized that activation domains with diverse structures may recruit different components of the basal apparatus or different classes of adapter molecules, thereby effecting transcription through distinct mechanisms. Indeed, the acidic activation domain of VP16 interacts with $TAF_{II}40$ to mediate activated transcription (23), while the Gln-rich activation domain of Sp1 interacts with $TAF_{II}110$ (10).

Several sequence-specific transcriptional repressors have recently been identified, but their mechanisms of action are less well understood. Repression domains have shown little or no sequence similarity to each other, indicating that they may have different downstream targets (28, 39, 47, 52, 53). As with the activators, some repression domains contact members of the basal machinery and/or interact with adapter proteins with the characteristics of a corepressor (48, 54). A protein is considered to function as a corepressor for a specific transcription factor when it fulfills the following criteria: (i) it represses transcription when fused to a heterologous DNA-binding domain (DBD), (ii) it interacts with the repression domain of the transcription factor, (iii) its binding is sensitive to inactivating mutations in the repression domain of the transcription factor, and (iv) it potentiates the repression function of the transcription factor in a concentration-dependent manner.

One class of repressor proteins consists of the nuclear hormone receptors. Receptors such as thyroid hormone receptor (TR) and retinoic acid receptor (RAR) repress basal transcription in the absence of ligand (2, 5, 6, 20, 24). The recent cloning of a class of proteins which have the properties of a corepressor for TR and RAR has begun to shed light on the mechanism by which these receptors silence transcription. It is hypothesized that in the absence of ligand, TR and RAR bind to one of a family of corepressors that includes the nuclear receptor corepressor (N-CoR) (30) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT) (9). Upon ligand binding, the corepressor is released, relieving repression and permitting transcriptional activation. AF2, or τ4, the amphipathic α helix present in the E region of many nuclear hormone receptors (15), is necessary for releasing corepressor (3, 9) as well as activating transcription (4, 15, 17).

RevErb is an orphan receptor encoded on the noncoding strand of the thyroid receptor α (c-*erbA*α) gene (35, 42) and is induced during adipocyte differentiation (8). RevErb has no known ligand, and in fact it lacks the AF2 domain which is required for ligand-dependent transcriptional activation by thyroid, retinoid, vitamin D, and steroid receptors. We have previously shown that RevErb constitutively represses transcription when bound as a dimer to a specific subset of DR2 sites (27). The C terminus of RevErb, including the hinge region and heptad repeats (22), is sufficient for repression

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when bound to the Gal4 DBD. Using the repression domain of RevErb as bait in the yeast two-hybrid system, we have cloned the TR/RAR corepressor N-CoR and show that it has all of the functional characteristics of a RevErb corepressor. Interestingly, the domains in RevErb and TR that are necessary for interaction with N-CoR are distinct in their primary amino acid sequences. Thus, N-CoR provides a common downstream pathway for transcriptional regulation by nuclear hormone receptors with different repression domains.

MATERIALS AND METHODS

Two-hybrid screen. RevErb 376-614 was cloned into pGBT9 (Clontech) by inserting *Pvu*II-*Xba*I (filled) fragment into the vector *Sma*I site. *Saccharomyces cerevisiae* HF7c [*MAT***a** *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1-HIS3, URA3*::(Gal4 17-mers)$_3$-*CYC1-lacZ*] containing pGBT9-RevErb 376-614 was transformed with a 17-day mouse embryo library in pGAD10 (Clontech) and plated on SD medium lacking tryptophan, leucine, and histidine (containing 5 mM 3-aminotriazole). His$^+$ colonies exhibiting β-galactosidase activity in the filter lift assay were further characterized. To recover the library plasmids, total yeast DNA was isolated, electroporated into *Escherichia coli* HB101, and isolated on minimal medium lacking leucine and containing ampicillin.

Plasmid constructs for transfection. Cloning of full-length RevErb, *GAL4*: RevErb 376-614 (27), and pCMX-N-CoR (30) was previously described. The remaining *GAL4*-RevErb fusions were first cloned into the KPN1 site of the pCDM-GAL4 vector. The N-terminal deletions were made by using the following 5′ primers for PCR (the underlined nucleotides indicate the *Bam*HI site; the boldface nucleotides indicate a *Kpn*I site): 5′-CCC**GGTACC**GTGGATCCCCC ACCCACGTGTATGCAGC-3′ (390 to 614), 5′-GGC**GGTACC**CTGGATCCC GGCAGGGCAACTCAAAGAAT-3′ (407 to 614), and 5′-GCC**GGTACC**TGG GATCCCCTATGAACATGTACCCGCAT-3′ (419 to 614). These primers were used with the following 3′ primer to amplify DNA fragments from CDM-RevErb: 5′-CCG**GGTACC**ATGGATCCGCCGGCCGGGCGGGTCACTG-3′. The C-terminal deletions were made by using the same template, a 5′ primer (5′-CG C**GGTACC**GTGGATCCAGCTGCCACCAGTCCAACAGC-3′, and a series of 3′ primers 5′-CCC**GGTACC**GTGGATCCCATGTTGTTCAGGGTCCGCA-3′ [377 to 601], 5′-CCC**GGTACC**GTGGATCCCTTGGTGAAGCGGGAAGTCT -3′ [377 to 587], and 5′-CCC**GGTACC**GTGGATCCCTTCAGCACCAGAGCC CGAA-3′ [377 to 576]). The entire *GAL4*-RevErb fusion gene was then shuttled out of the CDM vector with *Hin*dIII and *Xba*I, blunt ended with Klenow enzyme, and ligated into a blunt-ended *Bam*HI site in the pSG5 multiple cloning site. The Rev DR2 and Gal4 reporter vectors have been described elsewhere (27).

Plasmid constructs for GST fusion proteins. Glutathione *S*-transferase (GST)–RevErb 20-292 has been described elsewhere (26); GST–RevErb 20-129 was made from the previous clone by digestion with *Hin*dIII and *Eco*RI followed by ligation of a double-stranded oligonucleotide containing a stop codon. The remaining in-frame GST-RevErb fusions were all cloned into the *Bam*HI site of the pGEX2T vector (Pharmacia). The constructs described above in CDM-Gal4 were excised with *Bam*HI. Additional N-terminal deletions were made by using the following 5′ primers for PCR with the template specified above: 5′-GACG GATCCCGAGACGCTGTGCGTTTTGG-3′ (common 5′ primer) (200 to 614), 5′-CACGGATCCTGCCACCAGTCCAACAGCAAC-3′ (376 to 614), and 5′-C CCGGATCCATCTGGGAGGATTTCTCCAT-3′ (432 to 614). These clones were amplified by using the common 3′ primer, 5′-CCGGGATCCGCCGGCC GGGCGGGTCACTG-3′. The GST–Rev 200-614 (AH/GG) mutant was made by using two rounds of PCR. Round 1 used the common 5′ primer with 5′-GC GTAGGTGAAGATCTCTCGACCGCCCCGGGCCACCTGGG-3′ and 5′-TG AGATCCCAGGTGGCCCGGGGCGGTCGAGAGATCTTCAC-3′ with the common 3′ primer; round 2 used products from round 1 as a template with the common 5′ and common 3′ primers described above.

GST–N-CoR 1744-2453 and 1944-2453 were made in pGEX2T by using the 3′ primer 5′-CCCGGATCCTCAGTCGTCACTATCAGACA-3′ and the 5′ primers 5′-GGCGGATCCAGGATCAGCTGCTGCTCCCGC-3′ (1744 to 2453) and 5′-CCCGGATCCACTGCAGCTAACTTCATAGA-3′ (1944 to 2453), which was also used to make GST–N-CoR 1944-2239 with the 3′ primer 5′-GGCGG ATCCAGAGTCACCTCCACCAGAAG-3′. This 3′ primer was used with the following 5′ primers to make additional N-terminal deletions: 5′-GCGGGATC CGAGGTGATAAGTCCCGCCAG-3′ (1990 to 2239), 5′-GCCGGATCCCCA TCTCCACAGCAACAGCC-3′ (2040 to 2239), and 5′-GCGGGATCCGCTTC TACTTCTACATTCCA-3′ (2090 to 2239). GST–N-CoR 2239-2453 and 2296-2453 were cloned into pGEX-JDK *Eco*RI and *Xho*I sites, using the following primers for PCR: 3′ oligonucleotide (5′-TTTTTTTTTCTCGAGTCAATAAAA ACCAAA-3′), 5′-GGAGGTGAATTCGATATGGCAGCT-3′ (2239 to 2453), and 5′-AAAGTTGAATTCCATGGTGTTGTC-3′ (2296 to 2453). All PCR products, mutations, and fusion junctions were confirmed by sequencing.

Constructs for in vitro translation. pCMX-RevErb (16) and pCMX-N-CoR (30) were previously described. N-CoR 1510-2453 was excised from pGAD10 with *Not*I, filled in with Klenow enzyme, and ligated to the pCMX-HA *Bgl*II site with blunt ends. pCMX-HA was constructed by ligation of a fragment containing the hemagglutinin tag into pCMX. Full-length TRα1 was cloned into the pCMX *Eco*RI site. All constructs were in vitro translated by using the Promega TNT kit with [^{35}S]methionine and T7 polymerase.

Protein binding assays using GST fusion proteins. GST fusion proteins were expressed in *E. coli* BL21 by induction with 0.5 mM isopropythiogalactopyranoside (IPTG) at 30°C. Proteins were isolated by cell lysis with lysozyme and detergent followed by sonication. GST beads (50 μl) containing the fusion protein were incubated at room temperature in a buffer containing 50 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid [HEPES; pH 7.9), 2 mM EDTA, 0.1% Nonidet P-40 (NP-40), 10% glycerol, 0.5% nonfat dry milk, and 5 mM dithiothreitol. Five microliters of in vitro-translated RevErb, TRα1, or N-CoR 1510-2453 was added to the beads. Binding was allowed to proceed for 1 h, and then the beads were washed four times in the same buffer. The bound proteins were eluted by boiling in 30 μl of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer and resolved by electrophoresis. In each experiment shown, the amount of input protein was 1 μl, i.e., 20% of that incubated with GST-beads. The GST fusion proteins were stained with Coomassie blue to ensure equal loading, and the bound proteins were visualized by autoradiography.

Interaction of GST-RevErb with N-CoR from cell extracts. Cell extracts were made from 150-mm-diameter dishes of 80% confluent 293T cells which were either unlabeled or metabolically labeled with 1 μCi of ^{35}S-labeled methionine-cysteine mix (NEN) for 8 h in 5 ml of methionine- and cysteine-free Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The cells were washed, collected in phosphate-buffered saline (PBS), resuspended in whole cell extract buffer (150 mM NaCl, 50 mM Tris [pH 7.4], 5 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mg of leupeptin per ml, 1 mg of aprotinin per ml [25]) (1 ml per plate), lysed for 30 min at 4°C on a rotator, and cleared by centrifugation. Interaction assays were carried out by preincubation of GST or GST-Rev 200-614 (~6 to 8 μg) with 70 μl of glutathione-Sepharose beads followed by washing in buffer H (20 mM HEPES [pH 7.7], 50 mM KCl, 20% glycerol, 0.1% NP-40 [34]). Extract from half of a ^{35}S-labeled plate or 3 mg of extract from an unlabeled plate was added to the beads, and the samples were incubated at 4°C on a rotator for 1.5 h. Following centrifugation, the supernatant was removed and the beads were washed with 1 ml of ice-cold buffer H three times (unlabeled extracts) or five times (labeled extracts). The bound proteins were eluted by boiling in 1× SDS loading buffer (1), separated by SDS-PAGE, and transferred to nitrocellulose. The portion of the blot containing ^{35}S-labeled proteins was directly exposed to film, whereas the portion containing unlabeled proteins was probed for the presence of N-CoR by Western blot (immunoblot) analysis. Western blot analyses were done with a 1/350 dilution of primary guinea pig polyclonal N-CoR antiserum directed against amino acids 811 to 966 of murine N-CoR (30) and a 1/5,000 dilution of horseradish peroxidase (Sigma)-conjugated rabbit anti-guinea pig secondary antibody as previously described (32, 33) except that Tris buffer (50 mM Tris [pH 7.5], 140 mM NaCl, 0.1875% NP-40) was substituted for PBS-Tween in all solutions.

Cell culture and transfection. 293T cells were maintained and transfected in high-glucose Dulbecco modified Eagle medium with 10% fetal calf serum. At 80% confluence, 60-mm-diameter dishes were transfected by the calcium phosphate precipitation method, using 1 μg of luciferase reporter, 0.5 μg of β-galactosidase (β-Gal) expression vector, and 3 μg of receptor or corepressor expression vector (unless otherwise indicated in figure legends). Equivalent amounts of empty expression vector (pCDM, pCMX, or pSG5) were included in cells transfected with submaximal amounts of receptor or corepressor. Cells were lysed in Triton X-100 buffer, and β-Gal and luciferase assays carried out by using standard protocols (27). The measured relative light units were normalized to β-Gal activity, which served as an internal control for transfection efficiency. Fold repression was calculated as the activity of a given reporter after transfection with control expression vector divided by the activity of the same reporter in the presence of RevErb expression vector. Figures show the results of representative experiments in which individual datum points were assayed in duplicate or triplicate, and the range or standard error of the mean, respectively, is shown. Each experiment was repeated two to five times. The degree of repression from a given site was highly consistent from experiment to experiment.

RESULTS

N-CoR interacts with the RevErb repression domain. The RevErb C terminus has been previously shown to include a potent transcriptional repression domain (27). To repress transcription, RevErb must communicate with the basal transcription apparatus either directly or via additional protein intermediates. We have found that exogenous RevErb C terminus abrogated repression by full-length RevErb bound to its DR2 response element in a concentration-dependent manner (data not shown; see Fig. 2B). This finding suggested that a titratable cofactor mediates RevErb repression. The yeast two-hybrid screen was used to identify putative RevErb corepressors. Using amino acids 376 to 614 of RevErb as bait, 4 million trans-

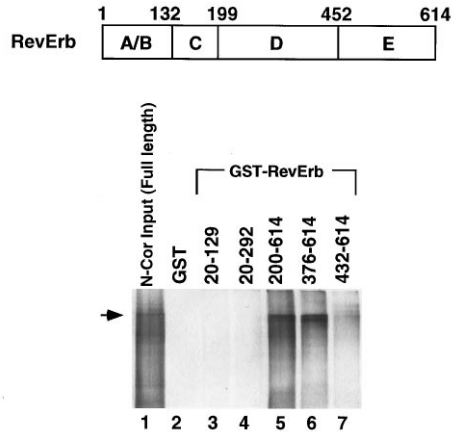

FIG. 1. N-CoR binds to RevErb in vitro. Full-length N-CoR was translated in vitro in the presence of [^{35}S]methionine and analyzed by affinity pull-down with the indicated GST fusion proteins bound to glutathione-Sepharose. The domains of RevErb are displayed at the top.

formants from a 17-day mouse embryo library were screened, yielding 84 interacting clones. One clone which was isolated three times was found to be identical to a recently described corepressor for TR and RAR called N-CoR (30). N-CoR is a 2,453-amino-acid (270-kDa) protein, and the partial clone which interacted with RevErb encoded amino acids 1510 to 2453. This clone contains the putative interaction domain of N-CoR (as well as another partial clone of N-CoR called RIP-13 [49]) which is also homologous to the interaction domain of a related nuclear receptor corepressor, SMRT (9). Figure 1 shows that GST-RevErb fusion proteins containing amino acids 376 to 614 specifically interacted with full-length N-CoR (lanes 5 and 6). In contrast, the RevErb N terminus and DBD did not interact with N-CoR (lanes 3 and 4). Furthermore, amino acids 432 to 614, which were previously shown to be insufficient for repression (27), were similarly unable to interact with N-CoR (lane 7).

N-CoR is involved in RevErb repression. If N-CoR is a true RevErb corepressor, then it should potentiate RevErb repression. This was tested in 293T cells. Figure 2A shows that N-CoR potentiated ligand-independent transcriptional repression by RevErb in a dose-dependent manner. N-CoR had no effect on transcription in the absence of cotransfected RevErb, consistent with its likely role as a cofactor which is recruited by DNA-binding proteins. The effects of N-CoR were modest (~2-fold) and required fairly high amounts of expression plasmid, most likely as a result of a combination of inefficient expression of the 270-kDa N-CoR protein and the expression of recombinant N-CoR in 293T cells which already contain endogenous N-CoR (see below and Fig. 3). The effect of N-CoR was specific, in that N-CoR did not potentiate the effects of the KRAB repression domain (39) fused to the Gal4 DBD (data not shown). These data suggest that N-CoR may be involved in mediating repression by orphan receptors in addition to TR and RAR. To further establish a role for N-CoR in repression by RevErb, we tested whether N-CoR could substitute for the titratable cofactor involved in RevErb repression. Gal4 fused to the RevErb C terminus was cotransfected along with full-length RevErb, resulting in a decrease in the ability of RevErb to repress basal transcription (Fig. 2B; compare lanes 2 and 3). The fusion protein includes the region of RevErb involved in N-CoR binding (amino acids 200 to 614). The decrease in repression is presumably due to squelching of a

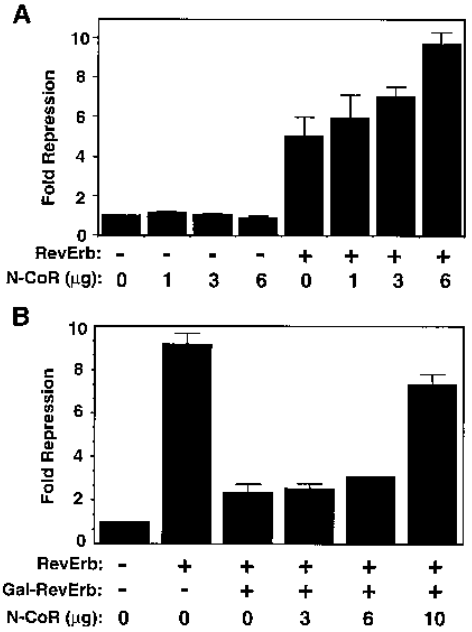

FIG. 2. N-CoR potentiates RevErb repression and can substitute for the endogenous corepressor in 293T cells. A RevErb expression plasmid was transfected into 293T cells along with a Rev-DR2–simian virus 40–luciferase reporter plasmid and increasing amounts of full-length N-CoR expression plasmid as indicated. (B) Same as panel A except that Gal4–RevErb 200-614 fusion protein was cotransfected as indicated.

titratable cofactor required for RevErb repression which was limiting in 293T cells under these conditions. As shown in Fig. 2B, lanes 4 to 6, increasing concentrations of N-CoR were able to relieve this squelching effect. Thus, N-CoR can potentiate RevErb repression and can substitute for the endogenous corepressor in 293T cells which is responsible for repression by RevErb.

RevErb interacts with endogenous N-CoR. To determine whether endogenous N-CoR in 293T cells interacted with RevErb, GST-RevErb was used to pull down interacting proteins in cell extracts from 293T cells. Figure 3A shows that after labeling of 293T proteins with [^{35}S]methionine, the C terminus of RevErb specifically pulled down a protein of ~270 kDa, the size of N-CoR. This was the only labeled species detected above 200 kDa, and no other proteins were clearly identified as being specifically associated with RevErb (although signal-to-noise limitations prevent strong conclusions about proteins smaller than 200 kDa in this experiment). To determine the identity of the 270-kDa RevErb-interacting protein, the GST pulldown experiment was repeated with unlabeled extracts, which were then subjected to Western analysis using a previously described antibody which recognizes N-CoR (30). As shown in Fig. 3B, Western analysis of the proteins pulled down by GST-RevErb identified a comigrating protein as being immunochemically related to N-CoR. A smear of somewhat smaller bands of lesser intensity was also noted; these bands likely represent breakdown products of N-CoR or other N-CoR-related proteins which specifically interacted with the RevErb C terminus. Comparison of N-CoR expression after transfection of the cDNA into these cells revealed little increase in level of the protein (not shown); however, since only 10 to 20% of cells were transfected, a 5- to 10-fold increase over endogenous levels would have been required to visualize a 2-fold change in the Western assay.

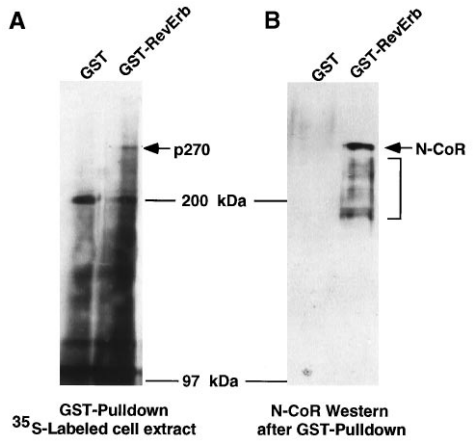

FIG. 3. Endogenous N-CoR interacts with RevErb. (A) A 270-kDa protein in 293T cell nuclear extract interacts with RevErb. Whole cell extract from ^{35}S-labeled 293T cells was incubated with GST or GST–RevErb 200-614 bound to glutathione-Sepharose. After washing, bound proteins were subjected to SDS-PAGE and autoradiography. (B) Endogenous N-CoR interacts with RevErb. Whole cell extract from 293T cells was prepared as for panel A from unlabeled cells. Extracts were incubated with GST or GST–RevErb 200-614 bound to glutathione-Sepharose. Bound proteins were subjected to SDS-PAGE and Western blot analysis with an antiserum which recognizes N-CoR (see Materials and Methods). Additional reactive bands below the major band are indicated by a bracket.

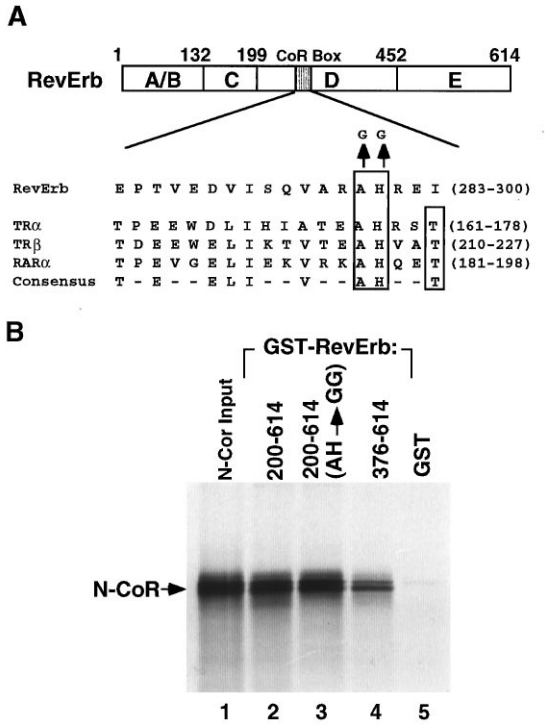

FIG. 4. The region necessary for TR and RAR interaction with N-CoR is not required for RevErb. (A) Conserved region of human TRα, TRβ, RARα, and RevErb. The position of the CoR box within the D domain is shown. Mutations of the boxed AHT abolish N-CoR interactions of TR and RAR (30), and the two amino acids mutated to G in RevErb are indicated. The P at 162 of human TRα is also found in v-ErbA and is required for repression as well as corepressor interaction (9). (B) N-CoR 1510-2453 was translated in vitro in the presence of [^{35}S]methionine and analyzed by affinity pull-down with the indicated GST fusion proteins bound to glutathione-Sepharose.

The RevErb interaction with N-CoR does not involve amino acids homologous to those required by TR and RAR. Interaction of N-CoR with TR and RAR has been shown to require a conserved domain within the D region of the receptors termed the CoR box; mutation of the A, H, and T residues (boxed in Fig. 4A) in TR has been shown to abrogate N-CoR binding (30). The same region of the receptors is also necessary for interaction with the SMRT corepressor (9). Figure 4A shows that RevErb contains a homologous region between amino acids 283 and 300. However, the CoR box was absent from the region (376 to 614) used in the two-hybrid corepressor screen. Thus, we were initially quite surprised that we identified N-CoR in this manner. The results in Fig. 1 indicated that this region was not necessary for direct RevErb–N-CoR interactions in vitro, as amino acids 376 to 614 of RevErb were sufficient for N-CoR interactions (compare RevErb 200-614 in lane 5 with RevErb 376-614 in lane 6). It remained possible that the homologous region of RevErb was necessary for N-CoR interaction in the context of the downstream sequences. However, Fig. 4B shows that mutation to glycine of the amino acids in RevErb homologous to TRβ amino acids A-223 and H-224 had no effect on the ability of RevErb to interact with N-CoR (compare lanes 2 and 3). Thus, the region of TR and RAR required for N-CoR interaction could be deleted or mutated in the context of RevErb with little effect on RevErb interaction with N-CoR. Furthermore, this mutation in Gal4–RevErb 200-614 had no effect on the ability of RevErb to repress transcription in 293T cells (data not shown).

Two regions of the RevErb C terminus are necessary for N-CoR binding. We next sought to identify the region of the RevErb repression domain responsible for interaction with N-CoR. N- and C-terminal deletions in the amino acids sufficient for repression (amino acids 376 to 614) were made and expressed as GST fusion proteins in *E. coli*. Equal amounts of these proteins were tested for the ability to interact with N-CoR. Figure 5 shows that N-terminal deletion to amino acid 406 had no effect on the ability of RevErb to bind N-CoR (lanes 5 to 7). However, deletion of an additional 12 amino acids (to 418 [lane 8]) abolished the RevErb–N-CoR interaction. These amino acids required for RevErb to interact with N-CoR are designated region X in Fig. 5. Remarkably, C-terminal deletions of Rev-Erb identified a second region of

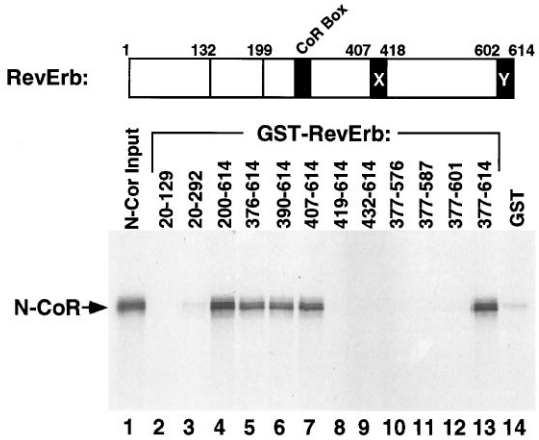

FIG. 5. Two separate regions in RevErb are required for N-CoR interaction. N-CoR 1510-2453 was translated in vitro in the presence of [^{35}S]methionine and analyzed by affinity pull-down with the indicated GST fusion proteins bound to glutathione-Sepharose. The regions of RevErb implicated in N-CoR binding are shown as box X and Y in the diagram at the top.

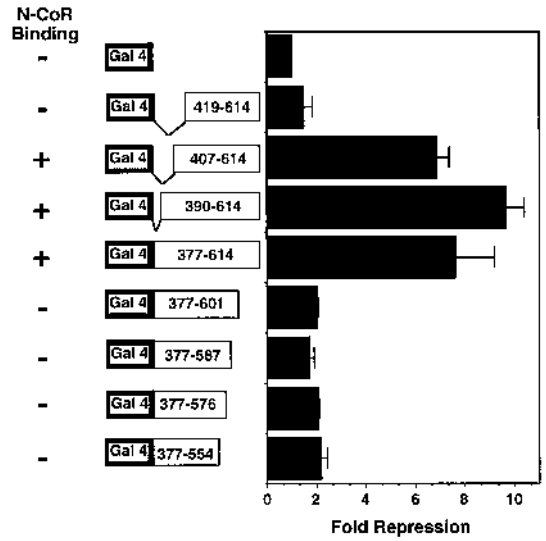

FIG. 6. N-CoR binding correlates with repression by RevErb. Regions of RevErb corresponding to those shown in Fig. 3 were fused to the Gal4 DBD and expressed in 293T cells along with a $(Gal4)_5$-simian virus 40-luciferase reporter plasmid. All of the Gal4 proteins were expressed at comparable levels, as determined by Western blotting with an anti-Gal4 antibody. The N-CoR binding of the polypeptides is summarized at the left.

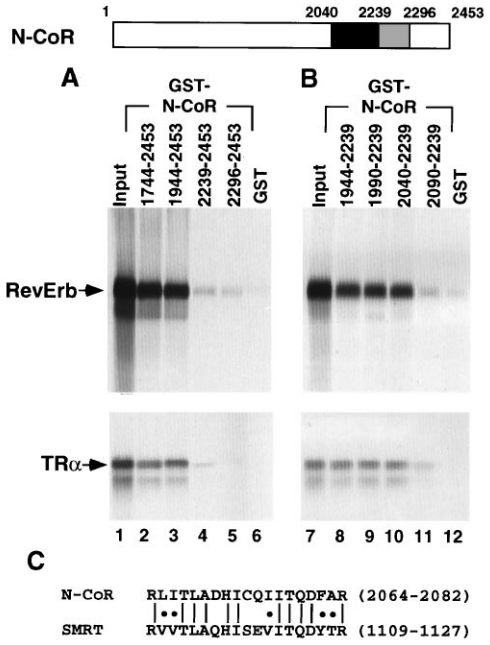

FIG. 7. Two domains in N-CoR interact with RevErb and TR. (A) Identification of a strong domain (1944 to 2453) and a weak domain (2239 to 2453). (B) Amino acids 2040 to 2239 are sufficient for strong interaction with RevErb and TR. Human RevErb and rat TRα1 were translated in vitro in the presence of [^{35}S]methionine and analyzed by affinity pull-down with the indicated GST fusion proteins bound to glutathione-Sepharose. The region implicated as a strong interaction domain for both TR and RevErb is indicated by the black box in the diagram at the top. The weak interaction domain which differentially interacts with TR and RevErb is shown as a lightly shaded box. (C) Homology between N-CoR and SMRT in this region. Dots indicate conserved but not identical residues.

RevErb required for N-CoR interaction. Deletion of the 13 amino acids at the C terminus of RevErb 602-614 also abolished N-CoR interaction. This region, Y in Fig. 5, is nearly 200 amino acids downstream from domain X. RevErb polypeptides which contained region X but not region Y (lanes 10 to 12) or which contained region Y but not region X (lanes 8 and 9) failed to bind N-CoR. Thus, domains X and Y are both necessary but not sufficient for RevErb to interact with N-CoR. Furthermore, both of these domains are distinct from the CoR box of TR and RAR.

The RevErb repression domain is identical to the N-CoR interaction domain. Thus far, we have shown that endogenous N-CoR interacts with RevErb, that N-CoR potentiates transcriptional repression by RevErb, and that N-CoR can substitute for a titratable cofactor necessary for RevErb repression in 293T cells. If endogenous N-CoR is responsible for RevErb repression, a further prediction is that RevErb mutants which do not bind N-CoR should not repress transcription in these cells. This hypothesis was tested by using the deletion mutants shown in Fig. 5, fused to the Gal4 DBD. Figure 6 shows that, indeed, RevErb polypeptides which included both the X and Y domains and hence bound to N-CoR in vitro (Fig. 5, 376-614, 390-614, and 407-614) strongly repressed transcription. By contrast, those that lacked either region X or region Y and did not bind N-CoR (419-614, 377-601, 377-587, and 377-576) had little if any ability to repress transcription. All of the transfected Gal4-RevErb fusions were expressed at similar levels, as determined by Western analysis using an antibody to the Gal4 DBD (data not shown). Thus, there was a strong correlation between the interaction of RevErb polypeptides with N-CoR and the ability of these polypeptides to function as repression domains.

Two domains of N-CoR differentially interact with nuclear receptors. Since two widely spaced regions of RevErb are necessary for interaction with N-CoR, and both regions differ from the TR domain, it was possible that N-CoR contains multiple domains which interact with RevErb and TR. To test this, a series of GST–N-CoR fusion proteins was constructed and the regions of N-CoR required for interaction with RevErb and TR were determined. We concentrated on the N-CoR C terminus since the interactions of N-CoR amino acids 1510 to 2453 with RevErb were indistinguishable from those of full-length N-CoR. Figure 7A shows that RevErb and TR both interacted strongly with amino acids 1944 to 2453 of N-CoR (lane 3). Using TR as a control, we examined the role of amino acids 2239 to 2296, which were previously shown to interact with TR in yeast cells (30). Lane 4 shows that the region from 2239 to 2453 was sufficient for weak binding of both TR and RevErb by N-CoR. Lane 5 indicates that amino acids 2296 to 2453 did not bind to TR, thus confirming that deletion of amino acids 2239 to 2296 abrogated TR binding. In contrast, this deletion did not affect RevErb binding (lane 5). Thus, this region of N-CoR differentially interacts with the two receptors, although the binding is relatively weak.

The more striking result in Fig. 7A is that deletion of amino acids 1944 to 2239 nearly abolished binding of both RevErb and TR to N-CoR (compare lanes 3 and 4). Indeed, Fig. 7B shows that this region of N-CoR was sufficient for strong nuclear receptor binding (lane 8). Additional dissection of this receptor interaction domain revealed that amino acids 1944 to 2040 of N-CoR were not necessary for strong binding of RevErb or TR (lanes 8 to 10). However, further deletion of amino acids 2040 to 2090 greatly reduced binding of both RevErb and TR (lane 11). Thus, amino acids 2040 to 2239 of N-CoR are sufficient to mediate strong binding to both RevErb and TR, and one or more motifs between amino acids 2040 and 2090 are necessary for these interactions. Interestingly, within this region is a 19-amino-acid stretch (amino acids 2064 to

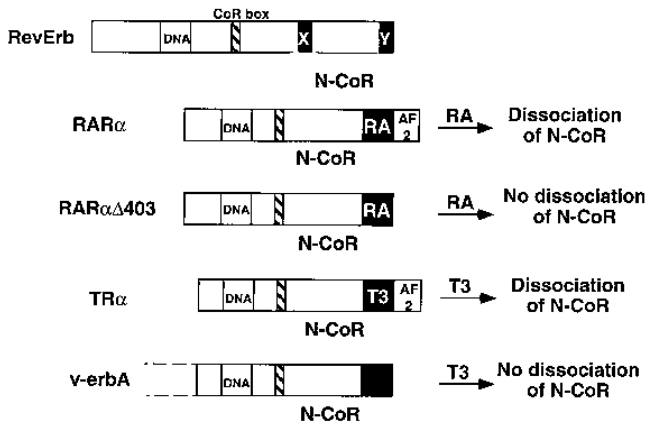

FIG. 8. Constitutive repression by RevErb and receptor mutants lacking AF2. RevErb functions as a constitutive repressor in a manner which is formally analogous to those of v-ErbA and the RARαΔ403 mutant, both of which bind corepressor in a ligand-independent manner as a result of a lack of AF2 function. The regions of RevErb which are required for N-CoR interaction are designated X and Y and shown as black boxes, as is the downstream region of TR and RAR. The region of RAR, TR, and v-ErbA required for N-CoR interaction (the CoR box) but not required in RevErb is shown as a striped box. These regions are indicated as interacting directly with N-CoR, although it is possible that one or more serve primarily a structural role (see text).

2082) with significant homology to amino acids 1109 to 1127 of another nuclear receptor corepressor, SMRT (Fig. 7C).

DISCUSSION

We have shown that N-CoR fulfills the criteria of a corepressor for RevErb. RevErb interacts with recombinant N-CoR in yeast cells and in vitro and interacts with endogenous N-CoR from mammalian cells. Preliminary data also suggest that this interaction can occur on DNA as well as in solution (57). N-CoR also potentiates RevErb repression and substitutes for a titratable cofactor involved in RevErb repression in 293T cells. Furthermore, the ability of RevErb mutants to bind N-CoR strongly correlates with their ability to repress basal transcription. Thus, N-CoR is a corepressor for RevErb in addition to TR and RAR.

In the cases of TR and RAR, ligand binding relieves repression by causing dissociation of N-CoR. Ligand binding also allows the receptors to interact with putative coactivator proteins such as ERAP140 and -160 (25), Trip1 (37), TIF1 (36), RIP140 (7), and SRC-1 (44). This function requires the AF2 domain. Significantly, RevErb lacks an AF2 domain, and in fact the protein terminates just at the point where the AF2 domain is found in ligand-regulated receptors. Thus, RevErb is likely not to be a direct activator of transcription, although it could conceivably activate transcription by interacting with a non-AF2-dependent coactivator or, indirectly, by competing with other receptors for corepressors. The AF2 domain also plays a role in release of corepressor from RAR. Truncation of RARα at amino acid 403 converts RAR into a constitutive repressor despite unaltered ligand binding (13). This is presumed to be due to the inability of ligand to induce dissociation of corepressor (11). Constitutive repression by RARαΔ403 is functionally similar to that by RevErb (Fig. 8). A similar mechanism explains constitutive repression by v-ErbA, which also has a defective AF2 (14). In all of these cases, the possibility of binding of a non-ligand-dependent coactivator simultaneously with the corepressor cannot be ruled out. Nonetheless, the question of whether RevErb has a ligand may be moot since the lack of AF2 makes it unlikely that binding of a ligand could activate or relieve repression by RevErb. This observation suggests that RevErb may be a prototype non-ligand-binding orphan member of the nuclear receptor superfamily.

The constitutive nature of RevErb silencing is likely to have functional significance. RevErb binds as a homodimer to a subset of DR2 elements which contain an AT-rich region upstream of the first half site (26, 27). RAR-retinoid X receptor heterodimers also bind to DR2 elements, from which they activate transcription in the presence of ligand (40, 43). Binding of RevErb to this subset of DR2 elements results in constitutive repression, while RAR-retinoid X receptor binding mediates ligand-dependent activation. One such naturally occurring DR2 occurs in the cellular retinol-binding protein I gene, which is activated by RAR (31, 50) and repressed by RevErb (27). This scenario allows for differential expression of genes with DR2-containing promoters, depending on the 5′ flanking sequence and spacer of the DR2 site. Thus, the activity of promoters containing these elements may be regulated by the availability of the corepressor in a cell type- and developmental stage-specific manner.

Two regions of RevErb, located nearly 200 amino acids apart and indicated as regions X and Y in Fig. 8, are required for N-CoR binding. When the RevErb C terminus is projected onto the crystal structure of the RARγ C terminus (45), these regions are actually quite close in three-dimensional space. Thus, it is possible that although they are widely spaced in the linear RevErb sequence, the X and Y regions form a single interaction surface. It is also possible that these regions represent the N- and C-terminal endpoints of one long interface. Further deletion mapping will be necessary to distinguish between these two possibilities. However, the two regions required for RevErb interaction with N-CoR are homologous to two regions of RevErbβ (16, 21, 46), which also binds N-CoR despite the fact that these domains are separated by a much shorter spacer (58). Thus, it is unlikely that the entire spacer region is required for N-CoR binding. An analogy may be drawn to p107 and Rb, which each contain two domains necessary for E1A-T antigen interaction. In p107, the two E1A-T antigen interaction domains are separated by approximately 200 amino acids, and cyclin A binds to p107 in the spacer region. Rb, in contrast, has a shorter spacer and does not bind cyclin A (18, 19). Thus, it is possible that other proteins bind to RevErb between the two N-CoR interaction domains, and additional clones isolated in the original dihybrid screen are being evaluated for this possibility.

Remarkably, the CoR box region in the D domain required for N-CoR interaction with TR and RAR is highly conserved in RevErb yet does not function in the same way. The homologous region of RevErb is not necessary for binding, since the polypeptide containing amino acids 376 to 614 of RevErb (which does not contain the CoR box) binds to N-CoR both in vitro and in yeast cells and is sufficient for transcriptional repression. However, although not crucial, this domain may contribute to N-CoR binding since the RevErb 376-614 polypeptide bound slightly less well to N-CoR than the RevErb 200-614 polypeptide (Fig. 4). Thus, it is possible that the CoR box plays a minor role in the RevErb–N-CoR interaction, although mutational analysis showed that the specific amino acids necessary in TR do not contribute detectably to the RevErb interaction (Fig. 4A).

We have identified two distinct regions of N-CoR which interact with nuclear receptors. The weak interaction domain corresponds to the region previously demonstrated in *S. cerevisiae* (amino acids) 2239 to 2296 (30). This domain is not required for maximum in vitro interactions with either RevErb

or TR. By contrast, the adjacent domain composed of amino acids 2040 to 2239 binds strongly to both receptors, indicating that the corresponding N-CoR interaction surfaces of RevErb and TR may have similar three-dimensional structures. Like RevErb, TR and RAR require at least two domains for repression function, including the CoR box in the D domain and a more C-terminal region located within the ligand-binding domain (2, 9, 30). Despite the dissimilarity in the primary amino acid sequences of the TR/RAR CoR box and the X domain of RevErb (Fig. 8), it is possible that both domains represent interaction surfaces which, in three-dimensional space, are equally recognized by N-CoR.

Alternatively, the CoR box, which is present in helix 1 of the TR and RAR ligand-binding domain crystal structures (45, 55), may serve a structural role. Conformation is clearly important for corepressor interaction with TR and RAR since release of N-CoR or SMRT is dependent on ligand. Thus, the CoR box in the D domain may function conformationally to expose a distinct interaction surface in the ligand-binding domain. RevErb has a unique and considerably larger D domain and thus may utilize a different structural mechanism, perhaps involving region X, to expose its N-CoR interaction domain. The domain directly involved in N-CoR interactions might be conserved among nuclear receptors. The Y domain required for RevErb repression is immediately adjacent to heptad 9 in the ligand-binding domain (corresponding to helix 10 in the RARγ crystal structure [45]) and is similar in sequence to this region of TR and RAR, which is required for repression (2, 6). In addition, amino acids 602 to 614 of RevErb have some homology to amino acids 917 to 928 of the progesterone receptor, which may be involved in antagonist-dependent repression (56). In conclusion, N-CoR functions as a corepressor for RevErb in addition to TR and RAR and is likely to function similarly with other members of the thyroid/retinoid/steroid receptor superfamily. These interactions are regulated by diverse primary structural determinants within the D domain of the receptors as well as by ligands.

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